Testprotocol for the shipboard verification of the ballast water treatment technology Coldharbour Marine Ltd.

Prepared for

Coldharbour Marine Ltd.

-- Confidential --

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Water and sediment of ships should be
Free of invasive organisms

There is no wisdom without ballast

Approved by	Quality Manager	Managing Director
Name / date	Name / date	Name / date

This report has been prepared under the MEA-nl Quality Management system. The Quality Management System in accordance with the ISO-9001:2008 standard of MEA-nl is certified by UKAS and periodically audited by Lloyd's Register.

Quality Management ISO 9001:2008

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1 INTRODUCTION

Shipboard Testing is a full-scale test of a complete Ballast Water Management System (BWM System) carried out on board a ship according to Part 2 of the annex to the Guideline G8 (Anon. 2008), to verify that the system meets the standards of regulation D-2 of the Convention (IMO, 2004).

Tests will be conducted as part of the certification process for Type Approval in accordance with the Ballast Water Management Convention 2004 (IMO, 2004) and relevant Guidelines. Lloyd's Register will witness the program and tests based on this test protocol on behalf of the Maritime & Coastguard Agency of the United Kingdom.

This test protocol aims to verify and document the performance characteristics of Cold Harbour Ltd BWM System in its task to prevent, minimize and ultimately eliminate the transfer of harmful aquatic organisms through controlling and managing ship's Ballast Water Sediments (BWM Convention, Article 2)

The ballast water Management procedures including treatment should be conducted as part of the standard procedures of the ship and carried out by the ship's crew according the operational and maintenance manual of the manufacturer.

The systems performance should be verified under normal operational procedures in water quality conditions which do represent natural conditions in terms of abiotic and biotic characteristics. In a separate test program (land-based testing) the BWMSystem should be tested under far more extreme conditions to guarantee a broad range of operational conditions.

This protocol is based on Guideline G8 (Anon, 2008) to the International Convention for the Control and Management of Ships' Ballast Water and Sediments 2004 (the Ballast Water Management Convention) of the International Maritime Organization (IMO) (IMO, 2004).

2 DESCRIPTION OF THE BALLAST WATER TREATMENT SYSTEM

The Coldharbour Marine (CHM) Ballast Water Management System (BWMS) is unique in the industry as it treats the ballast water in the tanks rather than in the line during ballasting. This offers many benefits to the operator, including no change to normal ballasting and de-ballasting operations, no additional power requirement during the ballasting/de-ballasting operation and the opportunity to top up the treatment during long voyages to tackle any regrowth of organisms prior to discharge.

The ballast water treatment system on-board M/V Alfa Glory is a unique in-tank system that utilizes an Inert Gas Generator (IGG) along with Gas Lift Diffusion (GLD) technology to kill invasive aquatic species within ships ballast tanks. Since this is a prototype installation, the control system is semi-automatic at this stage. The BWTS consists of the following principal components:

Combustion Air Blower, Inert Gas Generator, Fuel Oil Pumps, Instrument Air Compressor, Inert Gas Compressor, Gas Cooling Skid, Gas Lift Diffuser Pipe Assemblies, along with associated control and monitoring systems.

2.1 BWM System

The CHM BWTS is made up of a number of component systems:

- CHM 'Sea Guardian' Inert Gas Generator
- Process Gas Compressor
- Gas Lift Diffusers
- Micro Bubble Generators
- Ultrasonic Devices
- Process Pipework
- In Tank water condition instrumentation (full set in each ballast tank)

The systems, whilst standard in layout are individually designed to integrate with each other and the vessel requirements. The system described below is a full scale plant installed on the M/T Alfa Glory with the sole purpose of performing the sea based certification testing of the CHM BWTS. The system installed is indicative of the normal supply but restricted to No. 4 ballast tanks port and starboard and with some additional optional controls to allow additional data to be captured during the test period.

2.1.1 Inert Gas Generator

The CHM 'Sea Guardian' Inert Gas Generator (IGG), delivers ultra-clean, ultra-low O_2 gas for use with the companies BWT system. The IGG comprises of standard components detailed on the GA drawing IGG-21870 Rev 0. Operation is detailed in the user manual.

a. Combustion Air Blower

This standard roots type blower delivers the combustion air to the IGG and is the main volumetric flow. The Combustion Air Blower operation is detailed in the user manual.

Operating Parameters	Rated Flow	5000Nm3/hr
	Rated Pressure	up to 450 mbarG
	Motor Rating	90kW (VSD Control)

b. Fuel Pump System

This standard duty/standby fuel pump system delivers the fuel for the combustion process. The Fuel Pump System operation is detailed in the user manual.

Operating Parameters	Rated Flow	360l/hr
	Rated Pressure	up to 25 barG
	Motor Rating	0.5kW (each)

c. Inert Gas Generator

CHM standard 'Sea Guardian' IGG

Discharge Parameters	O_2	0.2% (+/- 0.2%)
	CO_2	12-14%
	Flow	up to 4500Nm^3/hr
	Pressure	up to 450mbarG
	Temperature	45°C Max (IGG Discharge)

2.1.2 Process Gas Compressor

The Process Gas Compressor (PGC) is a standard single stage oil free screw compressor, sized to match the IGG performance. The PGC has a indirect contact conditioning system to ensure that the gas delivered to deck is at the correct temperature.

Operating Parameters	Flow	up to 4100Nm^3/hr
	Pressure	up to 3.8 BarG
	Temperature	40°C Maximum
	Power	355kW (rated)

2.1.3 System pipework

The pipework used to connect each of the various components in the system is carbon or 316L stainless steel or GRE (glass reinforced elastomer) with cement joints.

2.1.4 Gas Lift Diffuser

The Coldharbour Marine Gas Lift Diffuser (GLD) is the means by which the water in the tanks is moved and treated. The GLD comprises of two treatment components that work in harmony with each other, but which are ineffective on their own. The two components are the Micro Bubble Generator (MBG) and the Ultrasonic Device (USD).

Materials of Construction	316L Stainless Steel/Duplex UNS31803

2.1.5 Micro Buble Generator

The MBG is a device that creates a volume of very small bubbles (<50µm) when supplied with a pressurised IG or air. The flow of IG or air into the MBG's is controlled by a diaphragm valve upstream of a flow meter.

Operating Range	~3.5Nm^3/hr of Inert Gas or Air per MBG
Materials of Construction	316L Stainless Steel/Duplex UNS31803

2.1.6 Ultrasonic Device

The USD is a device that uses Inert Gas or air to produce ultrasonic energy, that when exposed to micro bubbles causes them to implode, which results in a release of energy, killing the organisms on, or in close proximity to the bubble. The flow of Inert Gas or air is controlled by a diaphragm valve upstream of a flow meter.

Operating Range	Frequency	>20kHz
Sound Level	As high as is practical, typically >40dbA	
Materials of Construction	316L Stainless Steel/Duplex UNS31803	

2.2 Treated water

The water to be treated will have its pH and dissolved oxygen (DO) levels first lowered (deaeration) and then raised again (reaeration) during the process.

Operating Range	Deaeration	pH	<5.7
		DO	<0.6mg/l
	Reaeration	pH	5.9-6.3
		DO	>2.5mg/l

2.3 Treatment profile

One of the advantages of the Coldharbour Marine BWT system is that the treatment profile can be adjusted as the treatment is taking place. The treatment profile for the Coldharbour Marine BWT system is in four distinct phases:

1. Pre-Dwell The water is allowed to sit in the tank for a period of time, which mimics the real world reality that the system doesn't need to be switched on either during or immediately after ballast water is taken on board. During this phase the tanks can be stirred using air through the USD's ONLY. This phase is usually 2-3 days.

2. Deaeration In this phase IG is introduced through the MBG's and USD's to lower the DO and the pH to the aforementioned set points (as recorded by the in-tank instrumentation and fed back to the system control unit) whilst the interaction between the MBG's and USD's takes place. This phase is deemed to be complete when the DO has been stable at less than the set point for two hours. This period is typically around 12 hours, but can be longer or shorter depending on the condition of the intake water. In this way the system is able to mitigate the effects of varying intake water properties.

3. Dwell The water is allowed to sit in the tank in its deoxygenated state for the duration of the voyage until the time for next phase prior to de-ballasting.

4. Reaeration In this phase air is introduced through the USD's ONLY at a reduced flow to raise the pH and DO slowly to the aforementioned set points. This phase is usually completed within 8 hours

Note : In phase three the tanks can stirred using IG through the USD's ONLY to check that there are no pockets of air. This is normally carried out for 2-3 hours every other day.

3 DESCRIPTION OF THE SHIP IN RESPECT OF THE BWMS

The Coldharbour BWM System to be tested is installed on board the VLCC "*ALFA GLORY*". The vessel is managed by Gulf Marine of Hamburg and trading on the spot market.

3.1 General particulars

Ship's particulars	Ship's name	Alfa Glory
	Ship type	VLCC
	IMO number	9108154
	Built	1997
	Flag	Panama
	LOA	333 m
	GT	159422 mt
	DWT	309,636 mt
Ship Manager	Gulf Marine of Hamburg	

3.2 Ballast tanks on board

The ship is constructed with a ballast capacity of maximum 101/642,4 m3. The ballast water can be stored in 10 segregated ballast tanks and 4 tanks that may be used for ballast water. Details are given in Table 1.

Table 1: Ballast tank capacities

S.G = 1.025

COMPARTMENT	LOCATION (FR. NO.)	CAPACITIES		100% FULL		MAX. F.S.M (m4)
		VOLUME 100% FULL (CUB.M)	VOLUME 98% FULL (CUB.M)	L.C.G FROM A.P (M)	V.C.G ABOVE B.L (M)	
F. P. TK (C)	111 - F.E	4816.6	4937.0	311.835	9.461	16622
NO.1 W. B. TK (P)	101 - 111	8818.1	9038.6	279.990	11.471	61824
NO.1 W. B. TK (S)	101 - 111	8818.1	9038.6	279.990	11.471	61824
NO.2 W. B. TK (P)	91 - 101	9749.7	9993.4	228.751	9.460	102272
NO.2 W. B. TK (S)	91 - 101	9749.7	9993.4	228.751	9.460	102272
NO.3 W. B. TK (P)	81 - 91	9807.8	10053.0	178.050	9.411	103247
NO.3 W. B. TK (S)	81 - 91	9807.8	10053.0	178.050	9.411	103247
NO.4 W. B. TK (P)	71 - 81	9539.8	9778.3	127.684	9.623	96697
NO.4 W. B. TK (S)	71 - 81	9539.8	9778.3	127.684	9.623	96697
NO.5 W. B. TK (P)	56 - 71	8067.3	8269.0	76.845	12.146	39489
NO.5 W. B. TK (S)	56 - 71	8067.3	8269.0	76.845	12.146	39489
E/R W. B. TK (P)	16 - 35	1127.1	1155.3	22.521	23.591	675
E/R W. B. TK (S)	16 - 39	1711.7	1754.5	25.193	24.055	1077
A. P. TK (C)	A.E - 16	2021.6	2072.1	5.963	20.194	27151
T O T A L		101642.4	104183.5	-	-	-

Figure 1 gives a general arrangement of the ballast tanks on board.

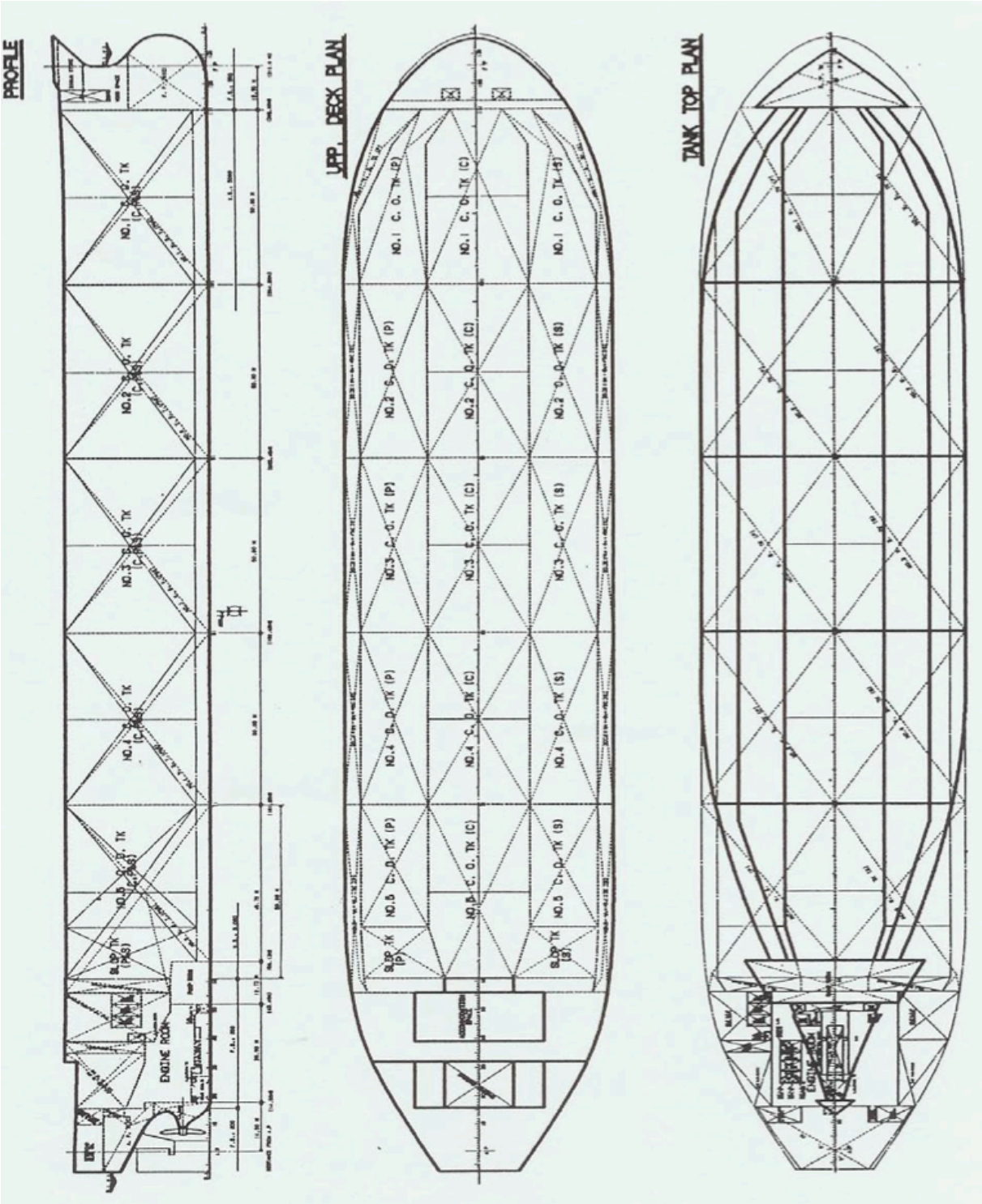

Figure 1: Ballast tank general arrangement

In Figure 2 details are given of the pump capacities. The ship can ballast and de-ballast up to 6000 m3/hr.

PUMP	LOCATION	CAPACITY
		M3 / HOUR
No.1 BALLAST PUMP	PUMP ROOM	3000
No.2 BALLAST PUMP	PUMP ROOM	3000
No.1 BALLAST EDUCTOR	PUMP ROOM	500
No.2 BALLAST EDUCTOR	PUMP ROOM	500

Figure 2: Ballast water pums

3.3 BWMS installed on board

The BWMS is a typical "in-tank" treatment system. For testing purposes, the tanks 4 starboard and portside are fitted with the tank-specific equipment. The system equipment is located inside a purpose built deckhouse situated adjacent to the port side engine casing.

For system operation throughout the trial period, the following information will be provided by Coldharbour Marine Ltd, separate from the test protocol:

1. Documentation of all ballast water operations including volumes and locations of uptake and discharge, and if heavy weather was encountered and where;
2. The possible reasons for the occurrence of an unsuccessful test cycle, or a test cycle discharge failing the D-2 standard should be investigated and reported to the Administration;
3. Documentation of scheduled maintenance performed on the system;
4. Documentation of unscheduled maintenance and repair performed on the system;
5. Documentation of engineering parameters monitored as appropriate to the specific system; and
6. Documentation of functioning of the control and monitoring equipment.

3.4 Piping and sample points

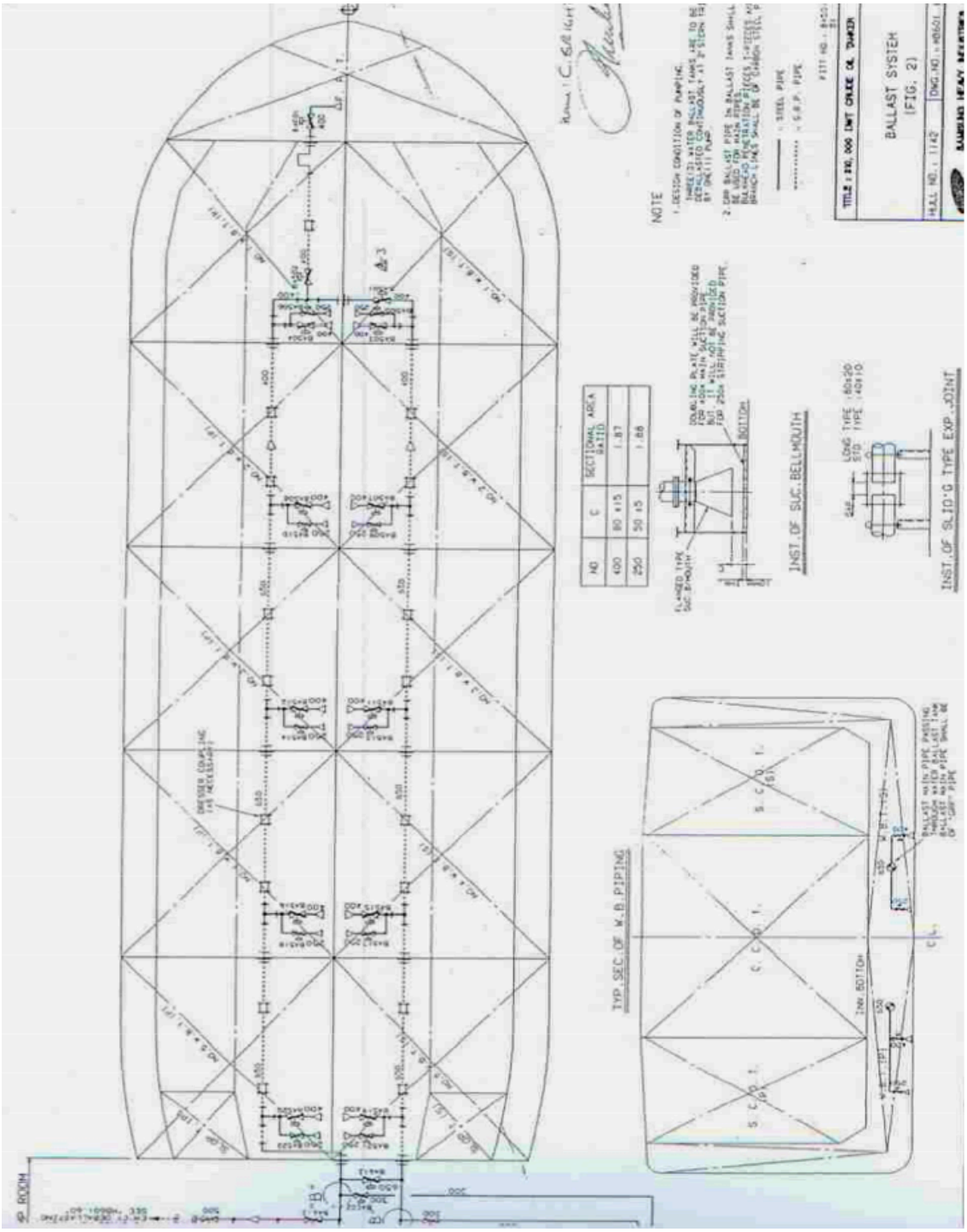

Figure 3: Plan of ballast piping

Figure 4 gives the sampling- and sounding points of the ballast tanks for Quarantaine officers. Sampling points are the manholes for entrance of the ballast tanks. Sounding points are the regular sounding pipes. Both cannot be used for the shipboard tests.

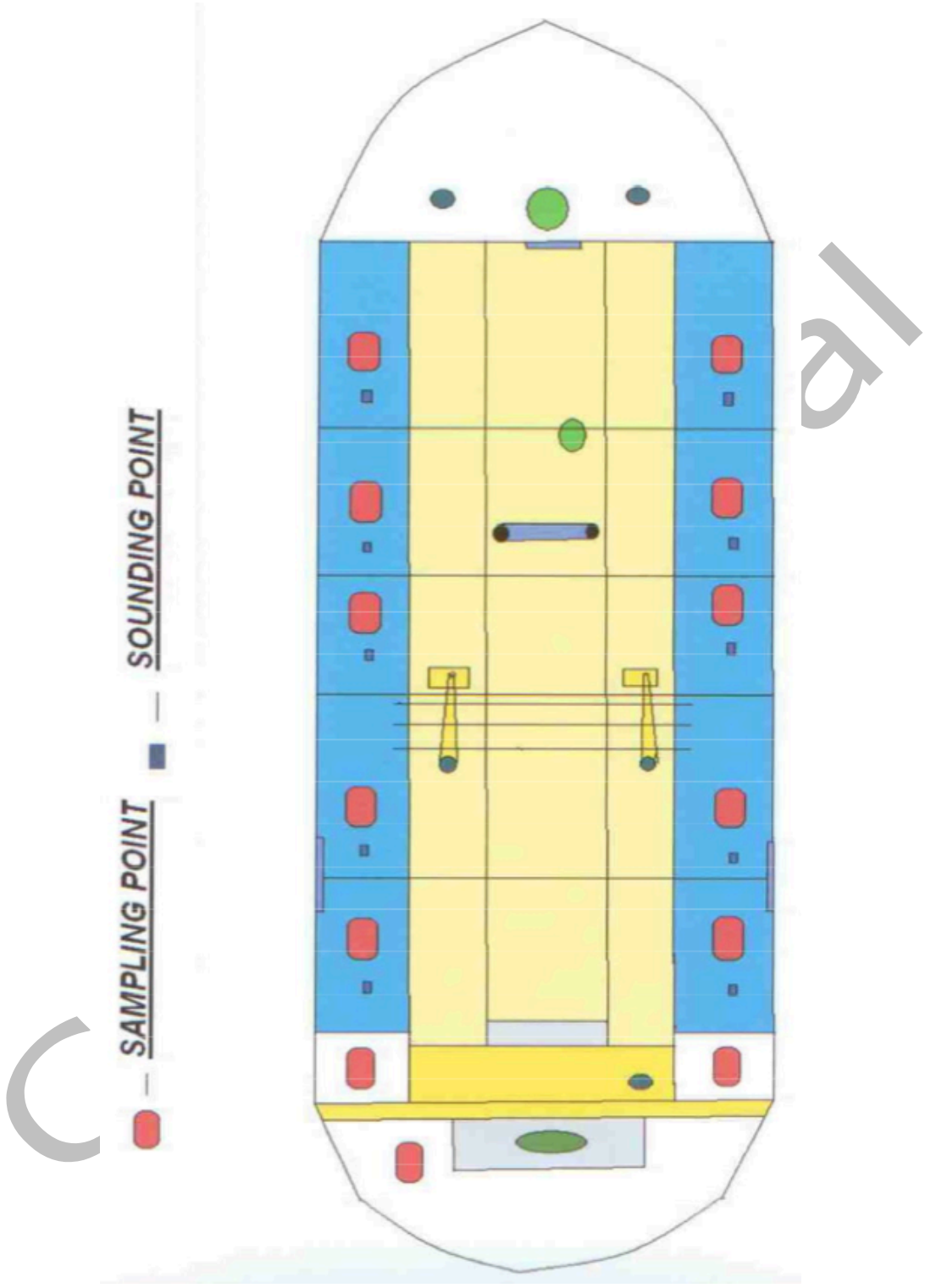

Figure 4: Sampling points for Quarantaine officers

A more detailed picture is given in Figure 5.

Figure 5: Manhole and sounding pipe of No. 5 WBT Starboard

4 TIMING AND LOCATION OF THE INTENDED TESTS

In order to perform successful shipboard tests, three valid test cycles in a six month period are required. These three test cycles have to meet the criteria for intake (par. 2.2.2.5) and for discharge (Regulation D-2) (IMO, 2004). Any invalid test cycle does not affect the sequence, but has to be reported. The test cycles including invalid and unsuccessful test cycles are to span a trial period of no less than six months.

Valid tests are indicated by uptake water, for both the control tank and ballast water to be treated, with viable organism concentration exceeding 10 times the maximum permitted values in regulation D-2.1 (IMO, 2004) and control tank viable organism concentration exceeding the values of regulation D-2.1 (IMO, 2004) on discharge.

The amount of ballast water tested in the test cycle on board should be consistent with the normal ballast operations of the ship and the BWM System should be operated at the treatment rated capacity for which it is intended to be approved.

The ship trades on the spot market, which makes locations for intake and discharge difficult to identify on forehand. Detailed description will be explained in the ship board test report.

5 PARAMETERS TO BE MEASURED

Part 2 of the Annex to the Guideline G8 (Anon., 2008) sets out the details for shipboard testing under par. 2.2. This is given in more detail in Annex 1.

The challenge conditions for ship board testing are specified into two groups of factors; general water quality conditions (abiotic factors) and numbers of living (viable) organisms as specified into different size classes.

5.1 Requirements for intake and discharge

The Guideline G8 (Anon. 2008), Part 2, Section 2.2.2.5 requires an uptake of organisms exceeding ten times the discharge standard. This translates into more than 100 viable organisms per cubic meter for the size class greater than or equal to 50 micron in minimum dimension. Bacteria and especially the indicator microbes (human pathogens) are not specified here. Requirements for those are specified in Section 2.2.2.6.3.3. The applicability of bacteria tests is limited by the storage time of the samples.

Upon discharge the treated water has to fulfil the requirements of Regulation D2 (IMO, 2004). The control water has to exceed the requirements upon discharge in order for the test run to be valid.

1. *less than 10 viable organisms per cubic metre greater than or equal to 50 micrometres in minimum dimension;*
2. *less than 10 viable organisms per millilitre less than 50 micrometres in minimum dimension and greater than or equal to 10 micrometres in minimum dimension; and*
3. *less than the following concentrations of indicator microbes, as a human health standard:*
   - *Toxicogenic Vibrio cholerae (serotypes O1 and O139) with less than 1 Colony Forming Unit (cfu) per 100 millilitres or less than 1 cfu per 1 gramme (wet weight) of zooplankton samples;*
   - *Escherichia coli less than 250 cfu per 100 millilitres; and*
   - *Intestinal Enterococci less than 100 cfu per 100 millilitres.*

Figure 6: Regulation D-2

5.2 Abiotoc parameters

The guideline, Part 2, Section 2.2.2.9 requires the following abiotic parameters to be measured for source water:

- Salinity
- Temperature
- Total Suspended Solids (TSS)
- Particulate Organic Carbon (POC)

These parameters will be measured at intake of the source water into the ballast tanks. Sampling and analysis will be done following the current SOP's as specified in Annex 4.

5.3 Biological parameters

The following biological parameters will be tested if they comply with regulation D2 of the Ballast Water Convention (IMO, 2004).

1. Viable organisms with minimum dimension equal to or larger than 50 microns
2. Viable organisms with minimum dimension between 10 and 50 microns
3. Indicator microbes belonging to the group of E. coli and enterococcus

Ad 2. Besides the size class of less than 50 micron and greater than or equal to 10 micron in minimum dimension, also phytoplankton smaller than 10 microns in minimum dimension will be analysed by PAM and FCM. This covers the whole range of biological relevant size classes. The group of organisms smaller than 10 micron in minimum dimension are of particular interest in the scope of Ballast Water Management because it includes species that can form harmful algae blooms. This is a deliberate deviation from the guidelines.

Ad 3. Total numbers of heterotrophic bacteria is determined for all samples. Because of travel time, applicability of human pathogens testing is limited in the scope the tests (Par. 2.2.2.6.3.3).

6 METHODOLOGY

6.1 Ballast process

TO BE WORKED OUT IN MORE DETAIL

Time required for bunker operations ……

Samples can only be taken when tanks will be filled or discharged completely and separately.

6.2 Sampling methodology

6.2.1 Sample locations

Sampling will be done at intake and discharge at appropriate locations. In practice this will be at intake prior to the ballast water tank and at discharge immediately before the sea-chest of the vessel.

6.2.2 Sample collection

All samples will be taken via a sample hose connected to an iso-kinetic sampling point in the ships ballast line. After sampling the water that is not needed will be pumped back into the main ballast line and discharged normally. If this is technically not possible, a suitable solution will be discussed with the chief engineer during the site visit and during the first (trial-)test run.

Sampling can be continuous or discrete depending on the parameter to be measured but in all cases cover the whole discharge period. In addition to water samples which are treated by the BWMS, water also water from a control tank, filled parallel at intake and discharge will be taken. All samples are taken to cover the whole intake and discharge process. Samples should be taken with minimal disturbance and additional stress to the organisms.

The sampling scheme described below for treated water differs from the guideline. There are two main reasons for this approach. Firstly the sampling strategy described in the guideline, Part 2, Section 2.2.2.6.2.1 is based on the assumption that subsamples from the sample are analysed. We will analyse the whole volume sampled. For analysis which involve subsampling (e.g. flow-cytometry) nine samples as described in the guideline will be taken. Secondly, by following the sampling strategy as described below a better comparability of results with the results from the land-based testing is given. This is due to follow the same sampling strategy for ship-board and land-based testing.

6.2.3 Abiotic parameters

Salinity and temperature will be measured at different intervals in discrete samples collected in a 12 Litre bucket (approx. 10 L) covering the whole pumping period.

Sub-samples for TSS and POC are collected in a 12 Litre bucket (approx. 10 L) and filtered immediately after sampling (pre-weighted GF/C). This volume depends on the particle load and will be between 200 and 1000 ml per filter. Sampling water in the

bucket will be mixed to assure dilution of particles prior to taking a sub-sample, since particles tend to sediment rapidly when stagnating. After filtration, volumes will be noted and filters will be put back into the same coded petri-dish and stored in a -20 °C freezer until further analysis.

6.2.4 Biological parameters

a. Plankton larger than or equal to 50 micron in minimum dimensions

Control samples
Control samples of 40 Litres into buckets and subsequently filtering this water over a 50 micron diagonal mesh (= 35 – 37 µm square). All organisms and material retained on the filter mesh will then be flushed into a clean sample bottle. Subsequent processing will be the same as for the treated samples.

Treated samples
Samples will be taken using a Hydrobios-plankton net with 50 micron diagonal mesh (= 35 – 37 µm square). The net will be suspended in suitable containers. The purpose of the containers is purely to volumetrically determine the sample size. However on board ship a portion of the water from the containers may be taken to prepare the organism free water necessary for sample processing. This will be done via a filtration cascade with an 0,2 µm filter (GF/C filter) as final step.

Samples will be taken over the whole pumping period. Individual samples should ideally be one cubic meter each, but practicality on board the vessel might dictate smaller volumes per sample. This will be determined in a technical test run prior to certification testing and will be documented as appropriate.

The content of the net will be flushed with organism free water into a clean sample bottle. The sample bottle will be stored in a manner to minimize adverse effects on the organisms and analysed as soon as practical.

b. Plankton smaller than 50 micrometer in minimum dimension

Samples will be taken separately for all protists (phyto- and microzooplankton). For the latter a bottle with 4 ml of Lugol's-solution will be filled with one litre of sample water directly from the sampling hose per sample. These bottles will then be stored in the dark until further analysed.
One litre samples will be taken for phytoplankton per sample directly from the sampling hose. This sample will be divided in various subsamples to be analysed immediately on board and/or at a later stage in the laboratory by PAM. One subset will be stored cool and dark (refrigerator or cooling box) until further analysed by flow-cytometry.

c. Human pathogens (indicator microbes)

Because of travel time, applicability of human pathogens testing is limited in the scope the tests (Par. 2.2.2.6.3.3). The outcome of the test may be affected in two different ways during a prolonged holding period. Firstly there may be on-going mortality of the human pathogens. Secondly, storage on board is not comparable with clean laboratory conditions, so samples may become contaminated, thereby introducing human pathogens. When practicable, samples will be analysed by an accredited lab accordingly.

d. Bacteria (total)

Subsamples for total bacteria numbers will be taken and preserved with glutaraldehyde or formaline, depending on availability. Final concentration for both fixatives will be 2%. Samples will be stored at -20 °C until transport. Transport will be in a cooling-box with cooling elements to assure that flow cytometric analysis can be done immediately after transport.

6.3 Sample processing

6.3.1 Abiotic parameters (SOP-306 and SOP-309)

Salinity and temperature will be measured using a standard multi-probe (Palintest Macro 900-system).

For TSS the filters are dried and weighed again. The concentration of TSS per litre can then easily be calculated from the sample volume and the weight difference of the filter before and after. TSS is expressed as mg per L.

For POC the filter is combusted at 550°C, allowed to cool and weighted again. The POC is calculated from the weight decrease between this measurement and the TSS weight. POC is expressed as mg C per L.

6.3.2 biological parameters

a. Plankton larger or equal to 50 micrometer in minimum dimension (SOP-320)

Control and treated samples
The content of the sample bottles will be analysed using a dissection microscope (a Leica MX 5 -or comparable- with cold light source is used). Samples will be analysed at 20x magnification as a standard for counting. Samples for this size-fraction will be always analysed entirely.

b. Plankton smaller than 50 micrometer in minimum dimension

Processing of samples for inverted microscopy (SOP-319)
Samples preserved with Lugols solution are analysed at a later stage. After arrival in the labsample bottles are stored in a dark for at least 24 hours. In this period all organic material will sediment due to the Iodium incorporated of Lugols solution. Sample bottles are concentrated and stored in a dark bottle until further analysis. Analysis of subsamples of the concentrate is carried out with an inverted microscope (method modified from Utermöhl 1958). Live-dead-separation in these samples is mainly based on the structural integrity of organisms.

Processing of samples for flow cytometry and PAM (SOP-317, SOP-318, SOP-322)
One litre samples will be taken directly from the sampling hose. Part of this sample will be stored cool and dark (refrigerator or cooling box) until further analysis by using flow-cytometry. Another part will be directly measured on board using a WALZ water-PAM to get a qualitative bulk measurement on phytoplankton vitality in the samples (Schreiber et al., 1993).

Flow cytometry:

Three replicate samples of 3 mL each will be taken from both control and treated water. They will be pipetted in an ultra-clean sampling tube and put in the carousel of a bench top flow cytometer (Beckman Coulter XL-MCL). As a light source a 15 mW Argon laser is used (488 nm excitation wavelength). Forward and side scatter is detected of each particle as well as the fluorescent emission in the yellow/green (525 ± 20 BP filter), orange (575 ± 20 nm) and red wavelength band (>645 nm, for details see Veldhuis & Kraay 2000). Samples will be counted using a standard protocol covering the particles in the size range of ca. 2 to 50 µm. Of all particles present in 1 ml of sample, cell size and presence or absence of chlorophyll a will be measured. Absolute numbers, cell sizes and chlorophyll a content of the particles will be analysed(for further details see SOP-318.

PAM fluorometry:
The photochemical efficiency of photosystem II (an indicator of the 'health' condition of the cell) of phytoplankton can be addressed using a Pulse-Amplitude Modulated fluorometer (PAM-fluorometry; Schreiber et al 1993). This simple parameter gives a qualitative indication of the photosynthetic activity of the phytoplankton community. For this 3 ml of unfiltered sample water (control and treated, each in triplicate) are filled into a glass cuvette and analysed using the Pulse-Amplitude Modulated fluorometer (automated measurement).

c. Bacteria

Total bacteria (SOP-316 and SOP-311)

The classical method for counting bacteria in many applications is based on plating at selective media. For aquatic samples of bacteria this approach is insufficient for various reasons (Gasol & Giorgio, 2000). Total bacteria will be determined by flow cytometry, using DNA-specific stains to get a more accurate bacteria number.

After complete thawing of the sample a subsample of 100 µL will be taken, diluted with a TE-buffer, and nucleic acid dye PicoGreen (Invitrogen) will be added. After a staining period of 5 to 15 minutes the sample will be analysed using a flow cytometer (cf. Gasol & Giorgio 2000; Veldhuis et al. 1997).

The dye PicoGreen is a green nucleic acid specific dye that only stains dsDNA, with little or no cross-over for ssDNA and RNA (Veldhuis et al, 1997). This makes the staining with PicoGreen method ideal for DNA staining and to determine bacterial abundance.

Human pathogens (SOP-311)

Because of travel time, applicability of human pathogens testing is limited in the scope the tests (Par. 2.2.2.6.3.3)(Anon., 2008). The outcome of the test may be affected in two different ways during a prolonged holding period. Firstly there may be on-going mortality of the human pathogens. Secondly, storage on board is not comparable with clean laboratory conditions, so samples may become contaminated, thereby introducing human pathogens.

When practicable, samples will be analysed by an accredited lab accordingly, e.g.:

Escherichia coli:
Analysis for coliforming bacteria and *coli* is carried out according to NEN-EN-ISO 9308-1, NEN 6570 and NEN 6571.

Enterococcus group:
Analysis for this group is carried out according NEN-EN-ISO 7899-2.

7 QUALITY ASSURANCE / QUALITY CONTROL (QA/QC)

7.1 Quality governance scheme

MEA-nl governs her processes and data by means of a system certified in accordance with the requirements of ISO-9001:2008. Scope of the certification is:

To perform tests for certification of ballast water management systems

June 6, 2013, the certificate is issued on behalf of the United Kingdom Accreditation Service (UKAS) by Lloyd's Register Quality Assurance.

This is laid down in the Quality Management Plan of MEA-nl. All analysis done at external labs are done conform the same quality requirements or higher. The present management system will be improved in accordance with the requirements of ISO-17025. This should be finished by September 2014 for MEA-nl.

MEA-nl also actively participates in the GloBal TestNet to develop standards for biological efficacy testing, to adhere to the latest developments and techniques and to perform inter-lab tests.

7.2 Project management

This test protocol is the Quality Assurance Project Plan (QAPP) for the shipboard tests. For the shipboard testing of the Coldharbour Marine Ltd. BWTS, Dipl.-Biol. F. Fuhr is assigned as the responsible project manager on behalf of MEA-nl.

7.3 Measurement and data aquisition

Quality of measurements and data acquisition is guaranteed by having a standard operating procedure (SOP) for every measurement needed to verify a BWT system. SOPs are part of our Quality Management System, assuring that measurements are done in the same way, with the same equipment, independent of which trained employee is performing the measurement. Methods are all based on well-known scientific procedures. Data are therefore always of the same quality.

All tests and measurements needed to verify a BWM System are done by experienced personnel. Personnel working from the start at MEA-nl is expected to have enough experience on methods they are responsible for. New personnel will be trained for every method they will perform on a base of: read-see-act (under supervision).

Test results will be recorded at forms attached in SOPs or in note books specified in the SOP. This assures that all data needed will be reported synchronously with the data collection. The verification strategy will be discussed in the next paragraph.

7.4 Verification of test data

Raw data generated during testing are important for the verification of the BWM System. Therefore these data will be recorded electronically and are stored on a regular basis. Electronic entry of raw data is the responsibility of the analyst/ scientist collecting the raw data and will be completed as soon as practically.

Electronic data are checked against the raw data by someone else at MEA-nl to assure the quality. Electronic data will be stored for at least seven years. Further details of data management can be found in BP-202 '*Data and Documentation management'*.

8 REFERENCES

8.1 Acknowledgements

8.2 List of references

Anonymous (2008) Guidelines for approval of ballast water management systems (G8). Annex 2 Resolution MEPC.174.58

Gasol JM, Giorgio PAD (2000) Using flow cytometry for counting natural planktonic bacteria and understanding the structure of planktonic bacterial communities. Sci. Mar. 64:197 - 224

IMO (2004) International convention for the control and management of ships' ballast water and sediments, 2004. Annex Regulation D-2. International Maritime Organization, London.

Schreiber U, Neubauer C, Schliwa U (1993) PAM fluorometer based on medium-frequency pulsed Xe-flash measuring light: A highly sensitive new tool in basic and applied photosynthesis. Photosynth. Res. 36:65 - 72

Shapiro HM (2003) Practical flow cytometry. John Wiley & Sons, Inc. New Jersey.

Veldhuis MJW, Cucci TL, Sieracki ME (1997) Cellular DNA content of marine phytoplankton using two new fluorochromes: taxonomic and ecological implications. J. Phycol. 33:527 - 541

Veldhuis MJW, Kraay GW (2000) Application of flow cytometry in marine phytoplankton research: current applications and future perspectives. Sci. Mar. 64:121 - 134

8.3 List of standard operation procedures (SOP's) of MEA-nl

SOP-306 Salinity, Temperature, pH, Dissolved Oxygen and turbidity
30-09-2013, version-number 1.1

SOP-308 sampling DOC and dissolved inorganic nutrients
24-01-2014, version-number 1.1

SOP-309 TSS, POC, MM and dissolved nutrients
26.09.2012; version-number 1.0

SOP-311 Human pathogens
24-01-2014, version-number 1.1

SOP-316 FCM Bacteria
26-09-2012, version-number 1.0

SOP-317 FCM Organisms 10 ≤ d < 50 micron
26-09-2012, version-number 1.0

SOP-318 PAM Measurements
26.09.2012, version-number 1.0

SOP-319 Micro zooplankton analysis
26.09.2012, version-number 1.0

SOP-320 Plankton sampling and analysis > 50 micron
26.09.2012, version-number 1.0

SOP-322 FCM Organisms < 10 micron in diameter
26.09.2012, version-number 1.0

8.4 List of figures

8.5 List of tables

8.6 Glossary of terms

Accreditation	The meaning assigned to it by Regulation (EC) No 765/2008.
Active Substance	A substance or organism, including a virus or a fungus that has a general or specific action on or against Harmful Aquatic Organisms and Pathogens.
Amendment	A change to a specific verification protocol or a test plan prior to performing the verification or test step.
Ambient Populations	The biological organisms, including bacteria, protists, and zooplankton that are naturally occurring in the water at the test facility location.
Ballast Water Management Plan	The document referred to in Regulation B-1 of the Ballast Water Management Convention (BWMC) describing the ballast water management process and procedures implemented on board individual ships.
Ballast Water Treatment Equipment	Equipment which mechanically, physically, chemically, or biologically processes, either singularly or in combination, to remove, render harmless, or prevent the uptake or discharge of Harmful Aquatic Organisms and Pathogens within Ballast Water and Sediments. Ballast Water Treatment Equipment may operate at the uptake or discharge of ballast water, during the voyage, or at a combination of such events.
Challenge Water	Water supplied to a treatment system under testing. Challenge water must meet specified ranges for densities of living organism and water-quality parameters and is used to assess the efficacy of the treatment equipment under full-scale operational conditions.
Cyst	The dormant cell or resting stage of microalgae, heterotrophic protists, and metazoans, including, but not limited to, cysts of dinoflagellates, spores of diatoms, cysts of heterotrophic protists, and cysts of rotifers.
Deviation	A change to a specified verification protocol or a test plan prior or during the verification or performance of a test step.
Effluent	The discharge of treated water produced by a ballast water treatment technology or system.
Equipment	The ballast water treatment system, defined as either a package or a modular system, which is to be tested for Type Approval.
General verification protocol (GVP)	The description of the principles and the general procedure to be followed by the ETV pilot programme when verifying an individual environmental technology.
In-Line Treatment	A treatment system or technology used to treat ballast water during a normal flow of ballast during intake or discharge.
Land-based Testing	A test of the ballast water management system (BWMS) carried out in a laboratory, equipment factory or pilot plant, either on land or on a test barge or test ship.
Manufacturer	A company or individual that manufactures, assembles, or sells ballast water management systems.
Monitoring Equipment	The equipment installed for the assessment of an effective operation of the Ballast Water Treatment Equipment.
Performance Data	Efficacy of removal and data on effluent concentration for core and supplemental parameters for a given set of challenge conditions.

Performance claim	A set of quantified technical specifications representative of the technical performance and the potential environmental impacts of a technology in a specified application and under specified conditions of testing or use (operational parameters).
Precision	The degree to which a set of observations or measurements of the same property, obtained under similar conditions, conform to themselves. Precision is usually expressed as standard deviation, variance, or range, in either absolute or relative terms (NELAC, 1998).
Protocol	A written document that clearly states the objectives, goals, scope, and procedures and logistics for the study of a particular group of similar technologies. A protocol shall be used for reference during manufacturer participation in the verification testing program.
Quality Assurance Project Plan (QAPP)	A written document that describes the implementation of quality assurance and quality control activities during the life cycle of the verification process (also see Test/quality assurance plan).
Representativeness	The degree to which data accurately and precisely represent a characteristic of a population.
Sensitivity	The capability of a test method or instrument to distinguish between different levels (e.g., concentrations) of a variable of interest.
Standard Operating Procedure (SOP)	A written document containing specific instructions and protocols to ensure that the requirements for quality assurance are maintained.
Test Cycle	One intake/discharge cycle (including appropriate holding periods) designed to gather data on treatment efficiency.
Test Facility	A site that provides the necessary infrastructure, equipment and (scientific) personnel to complete the land-based testing for Type Approval. The facility may be part of the Testing Organisation or may be independent from the Testing Organisation, but in any case shall be totally independent from manufacturers of technologies testing at their site.
Test/Quality Assurance Plan (TQAP)	Also called a Quality Assurance Project Plan (QAPP), is a written document that describes the procedures for conducting a test or study according to the requirements of the verification protocol for the application of a particular ballast water treatment system or technology. At a minimum, the TQAP shall include detailed instructions for sample and data collection, sample handling and preservation, precision, accuracy, goals, and requirements for quality assurance and quality control relevant to the particular site.
Testing Organisation (TO)	An organisation qualified to conduct studies and testing of ballast water treatment technologies in accordance with the appropriate protocols and TQAPs.
The Convention	The International Convention for the Control and Management of Ships' Ballast Water and Sediments (IMO).
Treatment Rated Capacity (TRC)	The maximum continuous capacity expressed in cubic metres per hour for which the BWMS is type approved. It states the amount of ballast water that can be treated per unit time by the BWMS to meet the standard in Regulation D-2 of the Convention.
Verification	Means the provision of objective evidence that the technical design of a given environmental technology ensures the fulfilment of a given performance claim in a specified application, taking any measurement, degree of uncertainty

	and relevant assumptions into consideration.
Verification Organisation (VO)	The party responsible for overseeing development of the TQAP, overseeing the testing activities in conjunction with the Testing Organisation, and overseeing the development and approval of the Verification Protocol, the Report and the Verification Statement for the ballast water treatment system. As certification is a task of a national administration, this is either a National Administration or a Classification Society authorised by the NA.
Verification Report	A detailed report on the testing results of a particular technology according to an approved Test /Quality Assurance Plan and conducted under the ETV/GTV protocol. The report is typically prepared by the Testing Organisation and contains a description of the test facility, photographs of the technology being tested, applied methods and procedures and a presentation of analysed data including all QA/QC data obtained during the test. Appendices include raw data sets and lab audit information, TQAP, O&M Manual and other relevant information.
Verification Statement	An executive summary of the verification report. A summary of the data will be part of Type Approval Certificate.
Verification Test	A complete test of a treatment system, following a well-defined TQAP which includes enumeration of ambient and test populations in the challenge water to determine the efficacy of the technology.
Viable	According to the IMO G8 Guideline, "organisms and any life stages thereof that are living". This differs to the scientific definition, "organisms which are capable of reproducing".
Vital	Essential to the continuation of life.

8.7 Abbreviations and acronyms

AC	Acetic acid
BE	Biological efficacy
DBP	Disinfection By-Products
BWM	Ballast Water Management
BWMS	Ballast water management system(s)
m^3	cubic meter, equivalent to 1000 Litres
DOC	Dissolved organic carbon
GESAMP	Joint Group of Experts on the Scientific Aspects of Marine Environment Protection (http//www.gesamp.org/)
GESAMP-BWWG	GESAMP-Ballast Water Working Group
GVP	General Verification Protocol (EU Environmental Technology Verification pilot programme
IMO	International Maritime Organisation (http//www.imo.org)
µg/L	Micrograms per litre
mg/L	Milligrams per litre
MM	Mineral Matter
n.d.	Not determined
n.a.	Not applicable
NEN-EN-ISO 9001	Quality Management Systems – Requirements (ISO 9001 2008)
NTU	Nephelometric turbidity unit
PAA	Per Acetic Acid
PSU	Practical salinity units
QA	Quality Assurance
QAPP	Quality Assurance Project Plan
QC	Quality Control
QMP	Quality Management Plan
SOP	Standard Operating Procedure
TA	Type Approval
TO	Testing Organisation
TQAP	Test/Quality Assurance Plan
TSS	Total Suspended Solids
VO	Verification Organisation

Annex 1 D-2 standaard uit de BWM Conventie

De huidige IMO norm voor het reinigen van ballastwater wordt gegeven in Regulation D2 van de Conventie. Het water dat wordt uitgepompt maximaal de volgende bestandsdelen mag bevatten:

- maximaal 10 levensvatbare organismen per m^3 die groter of gelijk zijn dan 50 µm; en
- minder dan 10 levensvatbare organismen per ml van minder dan 50 µm en groter of gelijk dan 10 µm.

Voor indicator microben zijn de volgende normen vastgesteld:

- toxicogenic *Vibrio cholerae* (O1 en O139) minder dan 1 cfu (colony forming unit) per 100 ml of minder dan 1 cfu per gram zooplankton;
- *Escherichia coli* minder dan 250 cfu per ml;
- Intestinal eneterococci minder dan 100 cfu per 100 ml.

Annex 2 Requirements for shipboard tests

The text hereunder is taken from par. 2.2 from part 2 of the Annex to the Guideline G8 (Anon., 2008).

2.2 Shipboard tests

2.2.1 A shipboard test cycle includes:

.1 the uptake of ballast water of the ship;

.2 the storage of ballast water on the ship;

.3 treatment of the ballast water in accordance with paragraph 2.2.2.3 by the BWMS, except in control tanks; and

.4 the discharge of ballast water from the ship.

Success criteria for shipboard testing

2.2.2 In evaluating the performance of BWMS installation(s) on a ship or ships, the following information and results should be supplied to the satisfaction of the Administration:

.1 Test plan to be provided prior to testing.

.2 Documentation that the BWMS is of a capacity within the range of the treatment rated capacity for which it is intended.

.3 The amount of ballast water tested in the test cycle on board should be consistent with the normal ballast operations of the ship and the BWMS should be operated at the treatment rated capacity for which it is intended to be approved.

.4 Documentation of the results of three consecutive, valid test cycles showing discharge of treated ballast water in compliance with regulation D-2.

.5 Valid tests are indicated by uptake water, for both the control tank and ballast water to be treated, with viable organism concentration exceeding 10 times the maximum permitted values in regulation D-2.1 and control tank viable organism concentration exceeding the values of regulation D-2.1 on discharge.

.6 Sampling regime:

.1 For the control tank:

.1 three replicate samples of influent water, collected over the period of uptake (e.g., beginning, middle, end); and

.2 three replicate samples of discharge control water, collected over the period of discharge (e.g., beginning, middle, end).

.2 For treated ballast water:

.1 Three replicate samples of discharge treated water collected at each of three times during the period of discharge (e.g., 3 x beginning, 3 x middle, 3 x end).

.3 Sample sizes are:

.1 For the enumeration of organisms greater than or equal to 50 micrometres or more in minimum dimension, samples of at least one cubic metre should be collected. If samples are concentrated for enumeration the samples should be concentrated using a sieve no greater than 50 micrometres mesh in diagonal dimension.

.2 For the enumeration of organisms greater than or equal to 10 micrometres and less than 50 micrometres in minimum dimension, samples of at least one litre should be collected. If samples are concentrated for enumeration the samples should be concentrated

using a sieve no greater than 10 micrometres mesh in diagonal dimension.

.3 For the evaluation of bacteria a sample of at least 500 millilitres should be taken from the influent and treated water. In the absence of laboratory facilities on board the toxicogenic test requirements should be conducted in an appropriately approved laboratory. However, this may limit the applicability of this test.

.7 The test cycles including invalid and unsuccessful test cycles are to span a trial period of not less than six months.

.8 The applicant is requested to perform three consecutive test cycles that comply with regulation D-2 and which are valid in accordance with paragraph 2.2.2.5. Any invalid test cycle does not affect the consecutive sequence.

.9 The source water for test cycles shall be characterized by measurement of salinity, temperature, particulate organic carbon and total suspended solids.

.10 For system operation throughout the trial period, the following information should also be provided:

.1 documentation of all ballast water operations including volumes and locations of uptake and discharge, and if heavy weather was encountered and where;

.2 the possible reasons for the occurrence of an unsuccessful test cycle, or a test cycle discharge failing the D-2 standard should be investigated and reported to the Administration;

.3 documentation of scheduled maintenance performed on the system;

.4 documentation of unscheduled maintenance and repair performed on the system;

.5 documentation of engineering parameters monitored as appropriate to the specific system; and

.6 documentation of functioning of the control and monitoring equipment.

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2.4 Reporting of test results

2.4.1 After approval tests have been completed, a report should be submitted to the Administration. This report should include information regarding the test design, methods of analysis and the results of these analyses.

2.4.2 The results of biological efficacy testing of the BWMS should be accepted if during the land-based and shipboard testing conducted as specified in sections 2.2 and 2.3 of this annex it is shown that the system has met the standard in regulation D-2 in all test cycles as provided in paragraph 4.7 below.

Annex 3 Sample analysis methods

Annex to the Guideline G8 (Anon., 2008), Part 4 – Sample analysis methods for the determination of biological constituents in ballast water

Sample processing and analysis

4.1 Samples taken during testing of BWMS are likely to contain a wide taxonomic diversity of organisms, varying greatly in size and susceptibilities to damage from sampling and analysis.

4.2 When available, widely accepted standard methods for the collection, handling (including concentration), storage, and analysis of samples should be used. These methods should be clearly cited and described in test plans and reports. This includes methods for detecting, enumerating, and identifying organisms and for determining viability (as defined in these Guidelines).

4.3 When standard methods are not available for particular organisms or taxonomic groups, methods that are developed for use should be described in detail in test plans and reports. The descriptive documentation should include any experiments needed to validate the use of the methods.

4.4 Given the complexity in samples of natural and treated water, the required rarity of organisms in treated samples under regulation D-2, and the expense and time requirements of current standard methods, it is likely that several new approaches will be developed for the analyses of the composition, concentration, and viability of organisms in samples of ballast water. Administrations/Parties are encouraged to share information concerning methods for the analysis of ballast water samples, using existing scientific venues, and papers distributed through the Organization.

Sample analysis for determining efficacy in meeting the discharge standard

4.5 Sample analysis is meant to determine the species composition and the number of viable organisms in the sample. Different samples may be taken for determination of viability and for species composition.

4.6 Viability of an organism can be determined through live/dead judgement by appropriate methods including, but not limited to: morphological change, mobility, staining using vital dyes or molecular techniques.

4.7 A treatment test cycle should be deemed successful if:

.1 it is valid in accordance with paragraph 2.2.2.5 or 2.3.36 as appropriate;

.2 the average density of organisms greater than or equal to 50 micrometres in minimum diameter in the replicate samples is less than 10 viable organisms per cubic metre;

.3 the average density of organisms less than 50 micrometres and greater than or equal to 10 micrometres in minimum diameter in the replicate samples is less than 10 viable organisms per millilitre;

.4 the average density of Vibrio cholerae (serotypes O1 and O139) is less than 1 cfu per 100 millilitres, or less than 1 cfu per 1 gramme (wet weight) zooplankton samples;

.5 the average density of E. coli in the replicate samples is less than 250 cfu per 100 millilitres; and

.6 the average density of intestinal Enterococci in the replicate samples is less than 100 cfu per 100 millilitres.

4.8 It is recommended that a non-exhaustive list of standard methods and innovative research techniques be considered.

Sample analysis for determining eco-toxicological acceptability of discharge

4.9 Toxicity tests of the treated water discharge should be conducted in accordance with

paragraphs 5.2.3 to 5.2.7 of the Procedure for approval of ballast water management systems that make use of Active Substances, as revised (resolution MEPC.169(57)).